Purification and Characterization of Heterologously Expressed Mouse CYP2A5 and CYP2G1: Role in Metabolic Activation of Acetaminophen and 2,6-Dichlorobenzonitrile in Mouse Olfactory Mucosal Microsomes

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ABSTRACT

Numerous environmental chemicals are known to cause tissue-selective toxicity in the olfactory mucosa in rodents (Bonnefoi et al., 1991; Dahl and Hadley, 1991; Genter et al., 1992; Gu et al., 1997). However, little is known about whether these compounds are also toxic in human nasal mucosa. To better predict the potential toxicity of environmental chemicals in humans and to determine the mechanism of tissue-selective toxicity, we sought to identify the enzymes responsible for their metabolic activation in the animal models exhibiting toxicity.

In mice, several cytochrome P450 [P450; the nomenclature used in this report is according to Nelson et al. (1996)] isoforms have been detected in olfactory mucosa, including CYP1A2, CYP2A5, CYP2E1, CYP2G1 and one or more isoforms of the CYP3A subfamily (Hua et al., 1997; Su et al., 1996; Gu et al., 1997; Genter et al., 1998). Of these, CYP2G1 is detected only in the olfactory mucosa (Hua et al., 1996), whereas CYP2A5 is expressed in the olfactory mucosa at levels much higher than in liver, kidney and lung (Su et al., 1996). Heterologously expressed mouse CYP2G1 is active toward testosterone and progesterone (Hua et al., 1997), which is consistent with findings with rabbit CYP2G1 (Ding and Coon, 1994). CYP2A5 is known to be the major coumarin 7-hydroxylase in mouse liver (Negishi et al., 1989) and also

ABBREVIATIONS: P450, cytochrome P450; b2, cytochrome b2; OM, olfactory mucosal; SF9, Spodoptera frugiperta; DCBN, 2,6-dichlorobenzonitrile; BSA, bovine serum albumin; IgG, immunoglobulin G; 4-NP, p-nitrophenol; AP, acetaminophen; 3-OH-AP, 3-hydroxyacetaminophen; GSH, glutathione; GS-AP, 3-glutathionylacetaminophen; GS-DCBN, glutathione conjugate of DCBN epoxide; HPLC, high performance liquid chromatography.
has activity toward other foreign chemicals, such as aflatoxin B1 and N-nitrosodiethylamine (Camus et al., 1993; Pelkonen et al., 1994).

In the present study, the roles were examined of CYP2A5 and CYP2G1 in the metabolic activation of two known OM toxicants, DCBN and AP. DCBN is a herbicide used for weed control. AP is a widely used therapeutic agent. Both DCBN and AP have been shown to cause OM toxicity in mice (Jeffery and Haschek, 1988; Brittebo, 1993; Deamer et al., 1994). DCBN does not cause toxicity in liver at the doses that cause OM toxicity (Brandt et al., 1990), whereas AP causes liver and renal toxicity in addition to OM toxicity (Placke et al., 1987; Jeffery and Haschek, 1988).

Metabolic activation of DCBN and AP was studied using purified P450s in reconstituted systems and in liver and OM microsomal preparations. Heterologously expressed mouse CYP2A5 and CYP2G1 were obtained and purified to electrophoretic homogeneity. Antibodies, generated with purified CYP2A5, and chemical inhibitors were used to demonstrate the prominent roles of the two isoforms in OM microsomal metabolism. In addition, the potential roles of CYP1A2 and CYP2E1 were ruled out in experiments with animals lacking the Cyp1a2 gene (Liang et al., 1996) or mice with elevated CYP2E1 levels after acetone treatment. Furthermore, to determine the relative importance of CYP2A5 and CYP2G1 in metabolic activation, the kinetics were examined of AP and DCBN metabolism by the purified P450s. Our results indicate that although both CYP2A5 and CYP2G1 are active toward DCBN and AP, CYP2A5 may play a greater role in OM microsomal metabolism of AP, whereas the relative roles of CYP2A5 and CYP2G1 in DCBN metabolism may be dose dependent, with CYP2G1 playing a more important role at low substrate concentrations.

Materials and Methods

Heterologous expression and purification of mouse CYP2A5 and CYP2G1. Baculovirus-mediated heterologous expression of mouse CYP2G1 in insect cells has recently been achieved (Hua et al., 1997). The bacterial expression vector for CYP2A5 was constructed as described previously for CYP2A4 (Sueyoshi et al., 1995). Escherichia coli cells expressing mouse CYP2A5 were cultured in 1 liter of LB medium containing 100 μg ampicillin, 0.2% glucose and 0.5 mM L-aminolevulinic acid. The cells were incubated in an orbital shaker at 37°C for 4 hr at 225 rpm before addition of isopropyl-β-D-thiogalactoside (Stratagene, La Jolla, CA) to a final concentration of 0.5 mM and were collected after a 48-h incubation at 25°C and at 150 rpm. The cell pellet from 1 liter of culture was washed in 40 ml of 20 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl and 5 mM EDTA and resuspended in 2 vol of the same buffer. The cells were lysed by three passes through a French Pressure Cell (SLM) using the high setting and a gauge reading of 600 to 700. Cell debris was removed by centrifugation at 9500 rpm for 10 min in a JA 20 rotor, and the supernatant was centrifuged again at 35,000 rpm in a Ti 45 rotor for 1 hr to obtain the membrane fraction. The membrane pellet was washed with 50 mM Tris-HCl buffer, pH 7.4, containing 0.8 M KCl and 1 mM EDTA, and resuspended in 4 ml of 10 mM Tris-acetate buffer, pH 7.4, containing...
Prior to HPLC analysis, all samples were dialyzed against distilled water. The retention times of the two metabolites were similar to the ones described previously (Park et al., 1991). The source of a monoclonal antibody to rat CYP2E1 (mAb 1–98–1) and preparation of polyclonal antibodies to rabbit CYP2A10/11 and CYP2G1 have been described in earlier studies (Park et al., 1986; Ding and Coon, 1990; Ding et al., 1991).

**Determination of catalytic activities.** Formation of DCBN-protein adduct and GSH conjugates of DCBN epoxide (GS-DCBN) was assayed as described recently (Ding et al., 1996) with use of a radiometric HPLC system (Ding et al., 1996). The contents of reaction mixtures and the incubation conditions are indicated in the legends to tables and figures. Protein adducts were precipitated with ice-cold acetone, washed repeatedly with sodium dodecyl sulfate (1%) to remove nonspecific binding, dissolved in 1 M sodium hydroxide and analyzed by liquid scintillation counting. The radioactivity in the washed precipitates was compared with that of the total reaction mixture for calculation of the rate of adduct formation. Formation of GS-DCBN was determined by radiometric HPLC as described previously (Ding et al., 1996).

**AP metabolism was assayed essentially according to Morgan et al. (1983) with some modifications in the contents of reaction mixtures as indicated in the legends to tables and figures. Reactions were initiated by the addition of NADPH to a final volume of 250 μl and were terminated after incubation at 37°C for up to 10 min by the addition of 125 μl of 3 M perchloric acid. HPLC analyses of AP metabolites were performed with a Waters μBondapak C18 column according to Harvison et al. (1988), with a radiometric HPLC system as described recently (Ding et al., 1996). Metabolites were detected by absorbance at 250 nm and by radioactivity measurements with 14C-AP as the substrate. Metabolites of AP were identified on the basis of comigration with standards generated in enzymatic reactions with rat CYP1A1 in a reconstituted system (data not shown), which was previously shown to metabolize AP to 3-OH-AP and GS-AP in the presence of added GSH (Harvison et al., 1988). The retention times of the two metabolites were similar to the ones described previously.
reported earlier using the same chromatographic conditions (Harvison et al., 1988). Formation of GS-AP was also confirmed by its detection by radioactivity measurement in experiments with 3H-GSH and unlabeled AP (data not given). Formation of AP-protein adduct was assayed as described above for DCBN metabolism with use of [ring-UL-14C]-AP (6.3 Ci/mol, from Sigma) as a substrate.

Quantification of 3-OH-AP and GS-AP was initially carried out by measuring radioactivity of individual peaks with an online radioactivity detector, and the amounts were calculated on the basis of percent recovery of total radioactivity. Subsequently, the ratios of UV absorbance at 250 nm to radioactivity of the two metabolites and that of AP were calculated by plotting the integrated peak areas from UV detector vs. those from radioactivity detector using samples from 10 experiments. In all cases, the linearity of the plots essentially extends through the origin (not shown). The slopes from these plots (60.4, 44.3 and 81.4 \( \mu \text{V} \cdot \text{sec}/\text{cpm} \) for AP, 3-OH-AP and GS-AP, respectively; \( r > 0.99 \) for all three compounds) were used to calculate the relative amounts of the two metabolites in reactions with unlabeled AP as substrate in later experiments.

Fig. 3. HPLC profiles of AP metabolites generated by CYP2A5 in a reconstituted system. Complete reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.6, a reconstituted system containing 0.2 \( \mu \text{M} \) CYP2A5, 0.6 \( \mu \text{M} \) NADPH-P450 reductase, 30 \( \mu \text{g} \) dilauroylphosphatidylcholine, 0.5 mM 14C-AP and 1 mM NADPH. Reactions were initiated by the addition of NADPH and were carried out at 37°C for 10 min (B and D). Control reactions (A and C) were terminated before the addition of NADPH. To determine GS-AP formation, GSH was added to the reaction mixtures at 10 mM (C and D). The metabolites were separated by reverse-phase HPLC and detected by UV absorbance (top: AU, absorbance unit) and radioactivity with an on-line radioactivity detector.

Spectral and electrophoretic characterization of purified, heterologously expressed CYP2A5 and CYP2G1. Bacteria-expressed CYP2A5 and SF9 cell-expressed CYP2G1 proteins were purified to electrophoretic homogeneity using conventional liquid-chromatographic methods after detergent solubilization of membrane preparations. As shown in figure 1A, a single band was detected by silver stain in purified preparations of CYP2A5 (lane 1) and CYP2G1 (lane 2). The two proteins migrated to about the same position and were detected as a single band when mixed (not shown). Efforts to resolve the two proteins by gel electrophoresis using different concentrations of acrylamide and by varying the length of the gels have so far been unsuccessful. For
shown) and absolute spectra of the cytochromes (fig. 1B) at 449 nm for both cytochromes with no peak at 420 nm (not carbonyl difference spectra exhibited maximal absorbance at 437 nm). The spin state of mouse CYP2G1 in the ferric form contrasts with that of CYP2A5 at 30 M substrate and higher for CYP2A5 at 30 M substrate but ≥20 times lower than that of OM microsomes. However, the addition of boiled mouse nasal or hepatic microsomes led to a substantial increase in the turnover numbers for both isoforms, whereas the addition of purified cytochrome b₅ led to further increases in the activities of CYP2A5 and CYP2G1 to about one half (for CYP2A5) and one third (for CYP2G1) of that found in OM microsomes. Metabolic activation of DCBN. The activities of purified CYP2A5 and CYP2G1 in metabolizing DCBN, an OM-specific toxicant, to DCBN-protein adducts were examined and compared with those of mouse liver and OM microsomes. As shown in table 1, OM microsomes were ≥20 times more active (per mg of microsomal protein) than liver microsomes in the formation of DCBN-protein adducts, with 33 to 82 times higher turnover numbers (per nmol P450) at 30 and 3 μM DCBN, respectively. The activities of the purified, reconstructed, cytochromes were initially examined with BSA added as a donor of sulfhydryl groups. With BSA present, both CYP2A5 and CYP2G1 were active toward DCBN, with turnover numbers similar at 3 μM substrate and higher for CYP2A5 at 30 μM substrate but ≥20 times lower than that of OM microsomes. Activity was not detected in control reactions containing boiled microsomes alone, with purified P450s omitted (not shown). These data indicate that both isoforms are active toward DCBN and that the dramatic difference in DCBN metabolic activities in liver and OM microsomes is not due to differences in the level of protein targets for adduct formation.

**TABLE 2**
Metabolic activation of AP by CYP2A5, CYP2G1 and mouse liver and OM microsomes.

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<td>OM microsomes</td>
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<td>(0.18 ± 0.03)^a</td>
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<td>Liver microsomes</td>
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^a Determined in the presence of GSH.
^b Values in parentheses indicate activities expressed as nmol/min/mg of microsomal protein.
^c BSA was added at 0.5 mg/ml.

reasons not understood, mouse CYP2G1 was not stained as intensely as CYP2A5 by silver, although the two proteins were stained to a similar extent by Coomassie blue (data not given); different amounts of protein were applied in figure 1 to yield similar intensities. The specific contents of P450 in the purified preparations were determined spectrally. The reduced carbonyl difference spectra exhibited maximal absorbance at 449 nm for both cytochromes with no peak at 420 nm (not carbonyl difference spectra exhibited maximal absorbance at 437 nm). The spin state of mouse CYP2G1 in the ferric form contrasts with that of CYP2A5 at 30 M substrate and higher for CYP2A5 at 30 μM substrate but ≥20 times lower than that of OM microsomes. However, the addition of boiled mouse nasal or hepatic microsomes led to a substantial increase in the turnover numbers for both isoforms, whereas the addition of purified cytochrome b₅ led to further increases in the activities of CYP2A5 and CYP2G1 to about one half (for CYP2A5) and one third (for CYP2G1) of that found in OM microsomes and much higher than activities seen in liver microsomes. Activity was not detected in control reactions containing boiled microsomes alone, with purified P450s omitted (not shown). These data indicate that both isoforms are active toward DCBN and that the dramatic difference in DCBN metabolic activities in liver and OM microsomes is not due to differences in the level of protein targets for adduct formation.

**Fig. 4.** Immuno-inhibition of OM microsomal AP and DCBN metabolism by anti-CYP2A5. Reaction mixtures contained 50 mM phosphate buffer, pH 7.4, 0.5 mM 14C-AP or 30 μM 14C-DCBN, OM microsomal preparation (0.2 mg protein/ml for AP or 0.1 mg protein/ml for DCBN), various amounts of anti-CYP2A5 IgG or preimmune IgG added to maintain a constant level of total IgG in all reactions (0.6 mg/ml for AP or 0.3 mg/ml for DCBN) and 1 mM NADPH. The mixtures were incubated on ice for 15 min and then at 37°C for 3 min before addition of NAPDH. Reactions were carried out at 37°C for 10 (for AP) or 30 (for DCBN) min, and protein adducts were quantified as described in Materials and Methods. The activities are shown as a percentage of rates determined in control reactions to which preimmune IgG alone was added. The values shown represent the average of two determinations with differences of <20% of the mean. The anti-CYP2A5 antibody inhibits both CYP2A5 and CYP2G1.

**Metabolic activation of DCBN.** The activities of purified CYP2A5 and CYP2G1 in metabolizing DCBN, an OM-specific toxicant, to DCBN-protein adducts were examined and compared with those of mouse liver and OM microsomes. As shown in table 1, OM microsomes were ≥20 times more active (per mg of microsomal protein) than liver microsomes in the formation of DCBN-protein adducts, with 33 to 82 times higher turnover numbers (per nmol P450) at 30 and 3 μM DCBN, respectively. The activities of the purified, reconstructed, cytochromes were initially examined with BSA added as a donor of sulfhydryl groups. With BSA present, both CYP2A5 and CYP2G1 were active toward DCBN, with turnover numbers similar at 3 μM substrate and higher for CYP2A5 at 30 μM substrate but ≥20 times lower than that of OM microsomes. However, the addition of boiled mouse nasal or hepatic microsomes led to a substantial increase in the turnover numbers for both isoforms, whereas the addition of purified cytochrome b₅ led to further increases in the activities of CYP2A5 and CYP2G1 to about one half (for CYP2A5) and one third (for CYP2G1) of that found in OM microsomes and much higher than activities seen in liver microsomes. Activity was not detected in control reactions containing boiled microsomes alone, with purified P450s omitted (not shown). These data indicate that both isoforms are active toward DCBN and that the dramatic difference in DCBN metabolic activities in liver and OM microsomes is not due to differences in the level of protein targets for adduct formation.

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^a Determined in the presence of GSH.
^b Values in parentheses indicate activities expressed as nmol/min/mg of microsomal protein.
^c BSA was added at 0.5 mg/ml.
Metabolic activation of AP. Purified CYP2A5 and CYP2G1 were found to be active in metabolizing AP to 3-OH-AP, and, in the presence of GSH, to GS-AP in a preliminary study (Genter et al., 1998). The activities of purified CYP2A5 and CYP2G1 in metabolizing AP to AP-protein adducts as well as 3-OH-AP and GS-AP were further examined here and compared with those of mouse liver and OM microsomes. The HPLC profiles of AP metabolites produced by CYP2A5 in a reconstituted system are shown in figure 3. The only soluble metabolite detected in the absence of GSH with either purified P450s or in liver and OM microsomal reactions (not shown) was 3-OH-AP. With the addition of GSH, GS-AP was detected as the predominant product in addition to 3-OH-AP in all reactions. Three additional, minor, peaks, which may represent unidentified products, were also detected (fig. 3D), with retention times of 4.0, 6.0, and 11.5 min, respectively; the small peak at ~11.5 min was also detected when 3H-GSH was used with unlabeled AP as substrate (data not shown), indicating that it may be a degradation product of GS-AP.

The turnover numbers of AP metabolism by purified P450s or microsomal preparations are shown in table 2. OM microsomes were 8 to 17 times more active (per mg of microsomal protein) than liver microsomes in the formation of the three products. The purified isoforms were also active in the formation of the two metabolites and AP-protein adducts, with turnover numbers higher than those found in liver microsomes. However, whereas the rates of formation of 3-OH-AP and GS-AP by purified CYP2A5 and CYP2G1 were close to those found in OM microsomes, the rates of protein adduct formation (per nmol P450) were 4 to 7 times lower in the reconstituted system, with or without (not shown) BSA present, than in OM microsomal reactions. In contrast to findings with DCBN-protein adduct formation, the addition of bovine or boiled liver or nasal microsomes did not change the rates of AP-protein adduct formation, and BSA had no effect on formation of 3-OH-AP and GS-AP (data not shown). In other experiments not presented, the effects of nondenatured microsomal fractions on AP-protein adduct formation were also examined. The addition of liver or nasal microsomes to reaction mixtures containing reconstituted CYP2A5 led to a small increase of overall activity, which, after deducting the activity seen in reactions with the microsomes alone, amounted to ~2 to 3 times of the activity seen in reactions with reconstituted CYP2A5 alone but was still lower than nasal microsomal activities.

Role of CYP2A5 and CYP2G1 in OM microsomal metabolism of DCBN and AP. The role of CYP2A5 and CYP2G1 in microsomal metabolism of DCBN and AP was examined with the rabbit anti-CYP2A5 antibody used in immunoblot quantitation. In experiments not shown, anti-CYP2A5 IgG completely inhibited coumarin hydroxylase activity of both CYP2A5 and CYP2G1 in a reconstituted system when added at 5 mg IgG/nmol P450. Thus, the effects of various amounts of anti-CYP2A5 IgG on microsomal metabolism of AP to AP-protein adducts and of DCBN to DCBN-protein adducts were examined, with preimmune rabbit IgG added to maintain a constant concentration of IgG in all reactions. As shown in figure 4, a concentration-dependent
inhibition of OM microsomal activities was observed with both substrates, with maximal inhibitions of ≥80% of control activities achieved at 3 mg IgG/mg microsomal protein, confirming that one or both of these isoforms play a major role in AP and DCBN metabolism in OM microsomes. The addition of preimmune IgG alone did not cause any inhibition of the activities (not shown).

The effects of two potential CYP2A5 inhibitors, 5-methoxsalen and 8-methoxsalen, on DCBN-protein adduct formation in reactions with CYP2A5 and CYP2G1 and with mouse OM microsomes were also examined. These compounds are known to inhibit the coumarin 7-hydroxylation activity in mouse liver microsomes (Maenpaa et al., 1993), and 8-methoxsalen has been found to inhibit heterologously expressed rat CYP2A3 (Liu et al., 1996). As shown in figure 5, A and B, the compounds inhibited the activity of the two isoforms as well as that of OM microsomes to a similar extent, suggesting importance of one or both of these isoforms in metabolic activation of DCBN. However, 8-methoxsalen is a more potent inhibitor than 5-methoxsalen; with substrate present at 3 μM, a 50% inhibition was achieved with ~2 μM 8-methoxsalen or ~10 μM 5-methoxsalen. Similar results were obtained with metyrapone (fig. 5C), a known inhibitor of DCBN toxicity in vivo (Brandt et al., 1990; Walters et al., 1993).

The effects of 8-methoxsalen and 5-methoxsalen on OM microsomal metabolism of AP to 3-OH-AP (fig. 6, A and B) and AP-protein adducts (fig. 6C) were also examined and compared with the effects on the purified isoforms (fig. 6, A and B). As was found for DCBN metabolism, both compounds inhibited the activity of the two isoforms as well as that of OM microsomes to a similar extent, suggesting importance of one or both of these isoforms in metabolic activation of AP. In other experiments not presented, several other compounds, including flavone, α-naphthoflavone, 4-methylpyrazole, 1-aminobenzotriazole and coumarin, were found to inhibit the activities of CYP2A5 and CYP2G1 to similar degrees and thus could not be used to determine the relative roles of the two isoforms in OM microsomal metabolism.

**Role of CYP1A2 and CYP2E1 in OM microsomal DCBN and AP metabolism.** The possible role of CYP1A2 in OM microsomal metabolism of DCBN and AP was examined with Cyp1a2(−/−) mice and wild-type littermates. As shown in table 3, rates of formation of AP- and DCBN-protein adducts were not decreased in OM S9 fractions from Cyp1a2(−/−) mice compared with Cyp1a2(+/+ ) mice, indicating that CYP1A2 does not play an important role in the metabolic activation of these compounds in the olfactory mucosa. For reasons not understood, S9 fractions from the CYP1A2-knockout mice had significantly higher activity toward DCBN than the wild-type littermates, although no difference was found with AP as a substrate.

The role of CYP2E1 was studied with mice treated with acetone to induce CYP2E1 level in the OM tissue. OM microsomes from acetone-treated mice had at least 3 times as much CYP2E1 protein as in microsomes from untreated mice, as demonstrated by immunoblot analysis with a monoclonal anti-2E1 antibody (data not shown). However, OM microsomal activities toward DCBN and AP were not different in the two groups (table 3). Thus, CYP2E1 does not play an important role in the metabolic activation of DCBN and AP in the olfactory mucosa. In other studies not presented, induction of CYP2E1 protein was not associated with an increase in OM microsomal activity toward 4-NP, a substrate frequently used for monitoring CYP2E1 activity in hepatic microsomes. Furthermore, both CYP2A5 and CYP2G1 were found to be highly active in 4-NP hydroxylation, with turnover numbers of 17.0 and 10.5 nmol/min/nmol P450, respectively, in a reconstituted system. Therefore, CYP2E1 may not play a major role in 4-NP metabolism and 4-NP hydroxylation may not be used for determining CYP2E1 induction in the olfactory mucosa.

**Kinetics of AP and DCBN metabolism by purified CYP2A5 and CYP2G1.** To evaluate the relative importance of CYP2A5 and CYP2G1 in OM microsomal metabolism of AP and DCBN, we determined the kinetics of AP and DCBN metabolism in a reconstituted system. As shown in table 4, CYP2A5 had only slightly lower \( K_{m} \) values than CYP2G1 did toward AP but much higher \( V_{max} \) values in the formation of both 3-OH-AP and GS-AP. With DCBN, however, CYP2G1 had a much lower \( K_{m} \) and lower \( V_{max} \) values than CYP2A5 did in the formation of GS-DCBN. Thus, it appears that CYP2A5 may play a greater role in OM microsomal metabolism of AP, whereas the relative roles of CYP2A5 and CYP2G1 in DCBN metabolism may be dose dependent, with
CYP2G1 playing a more important role at relative low substrate concentrations.

Discussion

Metabolic activation of DCBN is believed to go through 2,3- and 3,4-epoxy-DCBN, which form adducts with GSH or protein sulphydryls (Ding et al., 1996). Several P450 isoforms are known to be active toward DCBN, including rat CYP2A3, rabbit CYP2A10/11, rabbit CYP2E1, human CYP2A6 and human CYP2E1 (Ding et al., 1996; Liu et al., 1996). AP is metabolized either directly or through yet unidentified intermediates to 3-OH-AP and N-acetyl-metabolized either directly or through yet unidentified intermediates to 3-OH-AP and N-acetyl-p-benzoquinone imine; the latter is believed to be the toxic intermediate that reacts with GSH or protein sulphydryls to form covalent adducts (Hinson et al., 1980; Dahlin and Nelson, 1982; Harvison et al., 1988). Several P450s are known to catalyze this reaction, including human CYP1A2, CYP2E1 and CYP3A4 and their orthologs in other species (Morgan et al., 1983; Harvison et al., 1988; Patten et al., 1993; Lee et al., 1996; Kostrubsky et al., 1997; Zhou et al., 1997). However, definitive evidence for the P450 isoforms responsible for the metabolic activation of the two compounds in the olfactory mucosa has not been obtained in previous studies.

OM microsomes are much more active than hepatic microsomes in the metabolic activation of DCBN and AP, which may contribute to the tissue-selective toxicity of these compounds. Several lines of evidence were obtained in the present study that indicate that CYP2A5 and CYP2G1 play major roles in OM microsomal metabolic activation of DCBN and AP. First, purified CYP2A5 and CYP2G1 demonstrated activity toward the two toxicants in reconstituted systems. To this end, evidence was also obtained that CYP2A5 and/or CYP2G1 are abundant P450 isoforms in OM microsomes; the combined level of the two may account for >35% of total P450 in this tissue. Second, 5- or 8-methoxsalen provided parallel inhibition of AP or DCBN metabolic activation by the purified isoforms and OM microsomal preparations; similar results were obtained using an inhibitory anti-CYP2A5 antibody that also inhibits CYP2G1. In addition, the roles of two other P450 isoforms, CYP1A2 and CYP2E1, in the OM bioactivation of AP and DCBN were ruled out. There was no increase in the OM microsomal activities in acetone-treated mice compared with untreated mice, although OM microsomal CYP2E1 levels were 3-fold higher in acetone-treated mice. On the other hand, there was no decrease in the OM microsomal activities in Cyp1a2(−/−) mice compared with Cyp1a2(+/+) littermates. These results are consistent with a previous report suggesting that CYP2E1 may not play a major role in DCBN activation in rat OM microsomes (Eriksson and Brittebo, 1995) and with a recent study using Cyp1a2(−/−) mice indicating that CYP1A2 does not play a major role in the OM toxicity of AP (Genter et al., in press).

The effects of adding boiled liver or nasal microsomes on rates of DCBN- and AP-protein adduct formation in reactions with purified P450s are interesting. With DCBN, the addition of boiled microsomes instead of BSA as a donor of sulfhydryl groups led to a big increase in the rate of adduct formation. Similar increases were observed regardless of whether liver microsomes or nasal microsomes were used, indicating that the differences in liver and OM microsomal activities were not due to potential differences in microsomal proteins that can conjugate the reactive intermediates formed by P450 reaction. With AP, however, no increases were observed with addition of boiled microsomes. It remains to be determined whether the differential stimulation reflects different reactivity of the reactive intermediates formed from the two substrates.

The amino acid sequences of CYP2A5 and CYP2G1 are <60% identical (Hua et al., 1997). However, the two isoforms appear to be highly similar in immunological properties, substrate specificity and inhibitor selectivity. The two proteins are not resolved on immunoblots, and both react with several available antibodies prepared against CYP2A or CYP2G, including a monoclonal anti-CYP2A6 antibody from Gentest (data not shown). Both CYP2A5 and CYP2G1 are active toward a number of substrates, including coumarin, testosterone, progesterone, 4-NP, DCBN and AP, and both are inhibited similarly by all compounds tested to date. Furthermore, neither isoform appears to be inducible in the olfactory mucosa by chemical treatments. Although differences in kinetics parameters for DCBN and AP metabolism were found between purified CYP2A5 and CYP2G1, it is difficult at present to determine conclusively their individual roles in metabolic activation in OM microsomes. Attempts are being made to obtain isoform-specific antibodies to facilitate in vitro studies, and efforts to develop knockout mice models lacking either gene are warranted. The latter animal models will be very useful not only for resolving the role of the two P450 isoforms in microsomal metabolism but also for elucidating the role of these tissue-selective or -specific P450 forms in OM-specific toxicity of numerous foreign compounds, which is essential for reliable extrapolation of data from animal studies to risk assessment in humans.

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